Use of cell doublets for studying cytokinesis regulation reveals a new form of cytokinesis regression

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Article

Keywords: Cytokinesis regression, Cytokinesis index, Flow cytometry, Doublet discrimination cell-autonomous regulation, lymphocytic leukemia cells L1210

Posted Date: July 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-730080/v1>

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***Use of cell doublets for studying cytokinesis regulation reveals a new form of
cytokinesis regression***

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Running Title: Cell doublets and cytokinesis regression

Key words: Cytokinesis regression, Cytokinesis index, Flow cytometry, Doublet
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Abstract

Cytokinesis mediates separation of daughter cells at the end of cell division. We have developed a high-throughput approach for monitoring cell-autonomous cytokinesis in non-adherent cells. Focusing on cytokinesis termination, we show that chemical inhibition of protein phosphatase 1 (PP1) and PP2A specifically in late cytokinesis activates cytokinesis regression, which is distinct from any known cytokinesis failure, and is not a by-product of abnormal furrow ingression or chromatin bridges. This process is characterized by the formation of cortical blebs primarily at the intercellular bridge, reopening of the cleavage furrow and reassembly of an interphase-like microtubule network, but not by chromatin recondensation and mitotic spindle formation. Finally, cytokinesis regression is suppressed by chemical inhibition of aurora kinases but not Cdk1 or PLK1. Altogether, our results highlight a hitherto uncharacterized facet of the counter-activity of PP1/PP2A and aurora kinases in the final step of cell division, which ultimately secure the conclusion of cytokinesis, thereby preventing polyploidy and genomic instability.

Introduction

Scientists have been fascinated by the unidirectionality of the cell cycle ever since the pioneering era of cell biology¹. Cell cycle unidirectionality is largely dependent on orderly phosphorylation, dephosphorylation and ubiquitin-mediated proteolysis of regulatory and structural proteins². Mitotic entry is driven by the kinase activity of CDK1–cyclin B³⁻⁷. Following mitotic entry, proteolysis of cyclin B and inactivation of CDK1⁸⁻¹⁰, concomitantly with a massive dephosphorylation wave executed by protein phosphatases 1 (PP1) and PP2A and by more canonical cell cycle phosphatases (*e.g.*, CDC14) orchestrate cell division and mitotic exit into the G1 phase (G1) of the following cell cycle¹¹⁻¹⁹. This process is essential for successful cell proliferation.

Cell division ends with cytokinesis, in which the content of a mother cell is partitioned between two daughter cells. The physical division of the mother cell after chromosome segregation is first and foremost mediated by the contractile actomyosin ring, which ingresses a cleavage furrow at the cell equator. Furrow ingression ends with the formation of a dense filamentous structure known as the midbody^{20,21}. Cytokinesis is concluded when the intercellular bridge that connects the two sister cells is cut. This process is known as ‘cytokinetic abscission’ (abscission), is cell-cycle coordinated²²⁻²⁴ and integrates external cues from cell–cell and cell–surface contacts. These cues may, for example, involve forces that shape the dividing cell and the intercellular bridge in ways that influence the timing and mode of cytokinesis termination²⁵⁻²⁷.

Cytokinesis in unattached cells, such as lymphoblastoid cells, might be fundamentally different and more cell-intrinsic from cytokinesis in adherent cells, and consequently more uniform and predictable. However, animal cytokinesis has been almost exclusively studied in adherent cells. The reason for that is probably related to the high

demand for imaging of dynamic cytoskeletal and membrane structures that mediate cytokinesis. This level of imaging is undoubtedly simplified in adherent cells for reasons related to both optics and sample processing^{28,29}.

To study cytokinesis in a cell-autonomous manner, we developed a method for high-throughput flow cytometry analysis of cytokinesis in mammalian lymphoblasts growing individually in suspension. We discovered that short-term inhibition of PP1 and/or PP2A (PP1/PP2A) at the very end of cytokinesis induces acute furrow regression and polyploidy. This process is not an epiphenomenon of long-term manipulation, stalled cytokinesis, or chromatin bridges, and is driven by mitotic kinases. Our findings highlights an unappreciated role of protein dephosphorylation during cytokinesis in securing the unidirectionality of the cell cycle.

Results and Discussion

A new approach for systematic analysis of cytokinesis failure in non-adherent cells

We developed a pipeline for quantitative monitoring of cytokinesis failures in non-adherent cells. The method is based on applying short-term biochemical perturbations in L1210 mouse lymphoblasts using small-molecule drugs, and scoring of cytokinetic cells by optical flow cytometry. L1210 cells are spherical (axial ratio of 1.01), and have a remarkable tendency to grow only as singlets^{30,31}. These qualities are ideal for flow cytometry-based analyses, cell size measurements³²⁻³⁴, and for studying cell autonomous processes.

We established three complementary indices for detecting and scoring cells in cytokinesis, which we named 'phase index', 'doublet index' and 'size index' (**Figure 1 a-f**; see Supplementary information for more detailed information on the method). The phase index detects cytokinesis cells based on the cell cycle marker mAG-geminin³⁵ and on DNA

quantification. Cells with 4N DNA and basal expression of mAG-geminin are cytokinetic
(**Figure 1a, red gate**). This gating strategy was proven useful for purifying cytokinesis cells³⁰.
The doublet index relies on the optical resemblance between a late-cytokinesis cell, which
has fully ingressed cleavage furrow and a typical 8-shaped morphology, and a cell
doublet^{30,36} (note, cell doublets in flow cytometry are best detected by DNA
quantification³⁷). Because L1210 cells do not aggregate, an L1210 cell identified as a doublet
by flow cytometry is almost certainly a single cell in late-cytokinesis (**Figure 1b, grey gate**;
Panet et al., unpublished data co-submitted to *Communication Biology*). The size index
(**Figure 1c-d**) is based on the large size of cytokinetic cells (the size of L1210 cells is best
approximated by the pulse width of the forward light scatter (FSC-W)³³). Cytokinetic cells
can be distinguished from similarly large G2-phase and early mitosis (pre-anaphase) (G2/M)
cells by the mAG-geminin signal. Note that G2/M and G1 cells are indicated in all three
bivariate plots by black arrowheads (**Figure 1a-c, top plots**). Importantly, the size index
scores for two distinct size ranges named here 'F1' and 'F2' (**Figure 1c, dotted and straight
black gates**). F1 is an index for late-cytokinesis cells with fully ingresses furrow and 8-shaped
morphology (**Figure 1c, top image**). The F2 index captures cytokinetic cells with shape and
dimensions closer to that of G2/M cells (**Figure 1c, bottom image**). The size index
measurement is independent of chemical labeling. The potency of the three indices to
detect cytokines failures was validated in cells treated with pan-aurora kinase inhibitor VX-
680 (also known as Tozasertib). Aurora kinases are major regulators of cytokinesis^{20,21,38}
whose manipulation induces a variety of cytokinesis failures (**Figure S1**). All the cytokinesis
indices were informative and shifted in response to inhibition of aurora kinases (**Figure 1e**).
Importantly, these shifts followed different – sometime opposite – trends. Thus, the three
indices provide complementary information and highlight distinctive aspects on cytokinesis

failures induced by short-term chemical perturbations. Specifically, the phase index nearly doubled within 1 h of treatment. This trend is expected if cell division, but not mitotic exit, is halted. The end result is accumulation of cells with low mAG-geminin and with 4N DNA. The doublet index of cells treated only 1 h with VX-680 dropped by ~75%, mirroring a drastic reduction in the proportion of 8-shaped cells and the power of this new index. This result mirrors the extreme dependency of cytokinesis on aurora kinases. The size index was particularly informative. Both size indices F1 and F2 exhibited extreme shifts in cells treated 1 h with VX-680. While the F1 index dropped, reflecting a massive reduction in the frequency of 8-shaped cells (also observed by the doublet index), the fraction of cells excluded from size index F1 was added to size index F2. These results are well-aligned with cytokinesis failures that eventually lead to furrow regression and the formation of spherical polyploid cells, a stereotypical phenotype of VX-680 (**Figure S1**). Important, shifts in all indices were observed almost instantly even without computation, because of the inherently low number of cytokinetic cells, let alone late-cytokinesis cells, in an asynchronous population. This feature greatly simplifies data analysis.

Inhibition of PP1/PP2A in late cytokinesis induces cytokinesis regression

The phosphatases PP1 and PP2A are essential for the dephosphorylation wave that regulates mitotic exit and cytokinesis. The activity of PP1/PP2A can be blocked by the small molecule drug calyculin A (CaA)^{39,40}. We therefore tested whether CaA affects any of the four cytokinesis indices. Although the phase index was only modestly altered following 1 or 2 hrs exposure to CaA, the shift in the other indices was dramatic (**Figures. 1g-h, and S2**). The sharp reduction in the doublet index and size index F1 revealed a drop in the fraction of 8-shaped cytokinetic cells. Importantly, the noticeable reciprocal increase in size index F2

suggested that, rather than dividing, a significant portion of late-cytokinesis cells (F1 index) acquired a more spherical shape during incubation with CaA. Alternatively, the increase in size index F2 might have revealed a more global decoupling between mitotic exit (mAG-geminin proteolysis) and cytokinesis (cell division). Overall, the flow cytometry data presented in Figure 1 demonstrate that CaA-induced inhibition of PP1 and/or PP2A impairs cytokinesis.

PP1 and PP2A regulate every stage of the cell cycle, including cell division¹⁶. Chemical inhibition of PP1/PP2A blocks cell division (**Figure S3**)⁴¹. To examine the impact of CaA specifically on cytokinesis termination, we sorted L1210 cells using size index F1 as a gating strategy and obtained late-cytokinesis cells exhibiting a fully ingressed furrow and intercellular bridge (**Figures 2a and S4**), as well as decondensed chromatin and reassembled nuclei typifying cells at late telophase (**Figure 2b**). We refer to these cells as ‘late-cytokinesis cells’ from now on. To clarify, late-cytokinesis cells were isolated without chemical labeling, cell cycle arrest or any other means of pre-synchronization to allow direct and systematic manipulations of cytokinesis termination, thereby avoiding both manipulation of cells pre- and during furrow ingression and/or effects of external cues associated with cell-to-cell and cell-to-surface contacts.

We treated late-cytokinesis cells with CaA, hypothesizing that inhibition of PP1/PP2A at this stage will affect abscission. Admittedly, we did not foresee the outcome of this manipulation: the thin intercellular bridge puffed up, the furrow regressed completely, and the sister cells coalesced, generating a spherical polyploid cell (**Figure 2c and Supplementary movies 1-2**). This phenotype looked as if the entire process of cytokinesis proceeded backwards. We therefore named the process ‘cytokinesis regression’. Cytokinesis reversal is ubiquitous, dose-dependent and highly reproducible (**Figures 2d-e and S5**): at 50

nM CaA, 80% of the sister cells completed cytokinesis regression (%CR) and coalesced; lowering the dose of CaA to a 5 nM moderately reduced this value to 49%. This lower score was ideal for experiments discovering factors that either facilitate or repress the process. We established the 5 nM CaA treatment as the default protocol and limited image analysis to 3 h, which is when cytokinesis regression reaches a (near) maximum level.

Cytokinesis regression is not the result of cell fusion (**Figure S6**), nor is it dependent on cell sorting (**Figure S7**). Importantly, this phenomenon is not cell-type specific or even limited to non-adherent cells, as late-cytokinesis human epithelial HeLa cells exhibited cytokinesis regression following exposure to 5 nM CaA, with one noticeable difference — cell membrane blebbing⁴² was considerably more profound in HeLa cells relative to L1210 cells or any other lymphoblast cell line we tested (**Figures 2f-h, S7-9, and Supplementary movie 3**). Equally important, the PP1/PP2A inhibitor okadaic acid³⁹, but not the WIP1 inhibitor GSK2830371, also induced cytokinesis regression in both L1210 and HeLa cells (**Figures S10 and S11, and Supplementary movies 4**). Confocal microscopy images of L1210 cells with membrane-labeled green fluorescent protein (mem-GFP), improved visualization of membrane bulges and early events of cell membrane blebbing across the midbody zone (**Figure 2i, left top panel**). In addition, we used the docetaxel-based microtubule labelling fluorophore SiR-tubulin to image microtubule dynamics (as L1210 cells could not tolerate detectable ectopic expression of fluorescently tagged β -tubulin in our hands). Although using a taxol-based reagent for monitoring microtubule dynamics is conceptually problematic, this reagent allowed direct visualization of the CaA-induced deformation of microtubules along the intercellular bridge and visualization of the reorganization of an interphase-like microtubule network around the chromatin once cytokinesis regression concluded (**Figures 2i and S12**). Note that time resolution was greatly limited by

phototoxicity, in particular when z-stacks were required for 3D information and/or for detailed deconvolution of spherical objects with ~12-15 μ M/diameter.

Importantly, the chromatin of the coalesced sister cells remained decondensed (**Figure 2j**). Thus, PP1/PP2A inhibition in late cytokinesis induce cytokinesis regression, but not mitosis reversal⁴³. This result makes sense considering that cyclin B1 is already degraded at late telophase/cytokinesis and thus the kinase activity of the CDK1–cyclin B1 complex cannot be restored by inhibition of counter phosphatase activity. Because the nuclei of nascent sister cells are already reformed in late cytokinesis, we assumed that cells undergoing complete cytokinesis reversal should eventually end up being binucleated, which is a common phenotype of cytokinesis aberrations⁴⁴. To examine that, we monitored cytokinesis reversal in cells expressing the nuclear lamina marker mApple-lamin B1. Whereas HeLa cells undergoing cytokinesis reversal appeared binucleated (**Figure S13**), the labelling of chromatin and lamin B1 in L1210 cells suggested that the nuclei of the sister cells rearranged into a single polyploid nuclear structure (**Figures 2k-J and S13**). This process resembles sister nuclei coalescence, which is known in yeast⁴⁵, a closed mitosis organism, but not in mammalian cells. More in-depth live imaging and electron microscopy analyses, which are beyond the scope of this study, will be required to investigate the dynamics of the nucleus during cytokinesis reversal.

To summarize, in Figure 2 we show that PP1/PP2A inhibition in late cytokinesis triggers furrow regression followed by cell coalescence. This hitherto unknown process is not an epiphenomenon of long-term manipulations, abnormal cytokinesis or improper furrow ingression induced by chemical drugs or genetically^{41,44,46-51}, nor it is induced by chromatin bridges at the intercellular bridge (**Figure S14**) and the resulting ‘No-Cut’ response^{38,52}. These results highlight an unappreciated role for PP1/PP2A and overall

dephosphorylation in driving cytokinesis to termination. The enhanced blebbing activity associated with cytokinesis reversal likely indicates increased actin-myosin contractility⁴². It is noteworthy that CaA is known to intensify cellular contractility through regulation of myosin II^{53,54}, which is the major motor driving furrow ingression⁵⁵.

Cytokinesis regression is driven by aurora kinases

The prevalent understanding of the unidirectionality of mitotic exit is based on the premise that CDK1 activity cannot be restored following degradation of cyclin B, and that cell cycle phosphatases counteract the activity of mitotic kinases to regulate orderly proteolysis and other cascading events. Eventually, this cell cycle 'system' is reset before the cell is becoming committed to cycling again.

The cells we selected for the study of cytokinesis termination are at a very late stage of cytokinesis, and thus we did not expect inhibition of already-inactive CDK1 to induce cytokinesis regression by itself or to have any effect on CaA-induced cytokinesis regression. Indeed, incubation of late-cytokinesis cells with the selective CDK1 inhibitor RO-3306 did not induce cytokinesis regression (**Figs. 3a and S15**), and the effect of CDK1 inhibition on CaA-induced cytokinesis regression was almost negligible, albeit statistically significant (**Figs. 3b and S16, and supplementary movies 5 and 6**). These results suggest that remnant CDK1 activity associated with late cytokinesis may still be of some relevance to cytokinesis regression but, overall, when the phosphatase activity of PP1/PP2A is repressed, CDK1 is not the driving force of this process.

We hypothesized that other mitotic kinases are necessary for CaA-induced cytokinesis regression and tested the dependency of this process on aurora kinases and on

PLK1, which are key regulators of cytokinesis for which reliable small-molecule inhibitors are available. Aurora kinases and PLK1 are degraded during mitotic exit following cyclin B degradation and CDK1 inactivation, and their activity in late cytokinesis is undoubtedly considerable⁵⁶. We isolated late-cytokinesis cells and co-treated them with CaA and one of the following kinase inhibitors: VX-680 (inhibitor aurora A/B/C), AZD-1152 (aurora B) or BI2356 (PLK1). Both VX-680 and AZD-1152 strongly repressed CaA-induced cytokinesis regression (**Fig. 3c-d, 3f and S16, and supplementary movies 7 and 8**). The outcome of PLK1 inhibition was markedly different: BI2356 not only did not repress cytokinesis regression, it mildly, albeit consistently, enhanced it (**Fig. 3e-f and S16, and supplementary movie 9**). Note that neither BI2356 nor VX-680 or AZD-1152 induced cytokinesis regression on their own (**Figs. 3a and S15**). We concluded that cytokinesis regression induced by PP1/PP2A inhibition is repressed by inactivation of aurora kinases, but not PLK1. These results suggest that by the time cells are about to conclude cytokinesis and undergo abscission, they still possess an inherent ability to withdraw from cell division and block cell propagation. This ability is dependent on the activity of aurora kinases, and is prevented by PP1 and/or PP2A. The counteracting activities of aurora kinases and PP1/PP2A is well established (see for example Refs⁵⁷⁻⁵⁹). We speculate that the phosphatase activity of PP1/PP2A secures the conclusion of cytokinesis by directly repressing the activity of aurora kinases, and potentially of other mitotic kinases, through feedback mechanisms and/or through dephosphorylation of their key substrates during mitotic exit (**illustrated in Fig. 3g**).

The phenotype of cytokinesis regression, although regulated by the core machinery of mitotic exit, is fundamentally different from the mitosis reversal reported in the classic study by Gorbsky and Stukenberg⁴³. In their study, mitotic exit reversibility was achieved by

inhibiting proteasomal degradation of cyclin B or by expressing a non-degradable cyclin B. Mitotic cells advancing through mitotic exit upon drug-induced inactivation of CDK1 were able to re-enter mitosis, because cyclin B1 remained stable. Here, cytokinesis regression is induced considerably after the degradation of endogenous cyclin B has commenced. Indeed, we could not detect mitotic reversal in late-cytokinesis L1210 cells treated with proteasome inhibitors: treatment with 25 μ M of MG132 caused 0% cytokinesis regression ($N=107$), and treatment with 1 μ M bortezomib caused <1% cytokinesis regression ($N=370$) at $t=3$ h. These results suggest that at this advanced stage of cytokinesis, the mitotic activity of cyclin B1–CDK1 complex cannot be rescued by inhibition of proteolysis. Thus, we can say with confidence that the mechanisms of mitosis reversal and cytokinesis regression are different. Overall, we demonstrate an inherent decoupling between cytokinesis, i.e., the physical process of cell division, and the biochemical clock of the cell cycle. Inhibition of PP1/PP2A in late cytokinesis leads to the formation of a polyploid interphase cellular state, and not to the recovery of metaphase. Mechanistically, this decoupling can be achieved by PP1/PP2A regulation of phosphorylation sites that discriminate between cytokinesis and a more general mitotic function.

Cytokinesis regression is distinct from other forms of cytokinesis failure

Cytokinesis regression should not be confused with other forms of cytokinesis failure. This difference is particularly relevant for cytokinesis defects associated with manipulations of aurora kinases and their downstream targets. To clarify this point, we illustrate in **Fig. 4a** three possible fates of pro-metaphase cells facing chemical manipulations. Such cells can either divide normally or fail to initiate cytokinesis (cell division). Alternatively, the cells might commence cytokinesis, but fail to conclude furrow

ingression and midbody formation; eventually, the cleavage furrow reopens and a spherical polyploid cell is formed. L1210 cells at G2/M were isolated directly from an asynchronous cell population by sorting (see gating strategy in Figure. 1a, top right plot), and incubated with the aurora kinases inhibitor VX-680 or with DMSO (**Fig. 4b and c**). None of the G2/M cells treated with VX-680 divided: about half (54%) of the cells failed to enter cytokinesis, and the cells that did eventually commence cytokinesis (46%), failed to conclude furrow ingression, and re-acquired a spherical shape (**Fig. 4b and c, Fig. S17, and supplementary movie 10**). Despite the morphological resemblance, this failure is not the equivalent of what we define as cytokinesis regression, but a delayed response to an early mitotic perturbation of aurora kinases cause by VX-680. These results differentiate between aurora-related and PP1/PP2A-related phenotypes. No less important, they demonstrate the value of performing short and timing-specific perturbations when studying the cell cycle and especially cytokinesis: inhibition of aurora kinases blocks cytokinesis entry or interferes with cytokinesis progression if implemented early in mitosis (**illustrated in Fig 4e**). By contrast, inhibition of aurora kinases following furrow ingression, unlike inhibition of PP1/PP2A, does not prompt cytokinesis regression (**Figs. 4d, 3a and S15**).

To summarize, the discovery of CaA-mediated cytokinesis regression is yet another demonstration of the elaborate control of the unidirectionality of the cell cycle, in which PP1/PP2A activity is required for preventing polyploidy and maintaining genome and tissue integrity.

Small-scale and large-scale methodologies for studying cytokinesis are almost exclusively based on genetic or chemical manipulation of adherent cells, followed by automated imaging and advanced image analyses. We encourage colleagues who agree with us that mammalian cytokinesis must be studied also in non-adherent cells and in a

306 more cell-autonomous fashion to take full advantage of our new set of flow cytometry tools
307 developed for this purpose and others.

308

309 **Acknowledgements**

310 We thank Efrat Ozer, Assaf Bester and Michael Danilenko for reagents, and Manny
311 Benish, David Fradkin, Sharon Kum, Ofir Hakim and Avi Jacob for technical support. We are
312 also grateful to Jennifer Israel Cohen for biostatistics consultation and Marc Kirschner and
313 Michael Emanuele for critical reading. The Israel Science Foundation (ISF) is gratefully
314 acknowledged for funding; Grant no. 2038/19.

315

316 **Competing interests**

317 The authors declare no competing interests.

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Figure 1. A new methodology for systematic analysis of cytokinesis in non-adherent cells reveals cytokinesis failures associated with a short-term inhibition of PP1/PP2A. (a-f) Cytokinetic cells can be detected by three complementary flow cytometry indices. The method is demonstrated for mouse L1210 lymphoblasts, but is compatible with other cell types. The first index – ‘phase index’ – is based on the expression of the cell cycle marker geminin fused to monomeric Azami Green (mAG) fluorescent protein (mAG-geminin) and on DNA content. The expression levels of mAG-geminin during late cytokinesis and in G1 are minimal and so, cells with 4N DNA and basal mAG-geminin levels have passed anaphase but are yet to divide. These cells are, by definition, in cytokinesis (top panel, red gate). (b) The second index – ‘doublet index’ – relies on pulse width (W) vs. pulse area (A) of DNA signal, which is used for identifying cell doublets in flow cytometry analyses (top panel, grey gate). (c) The third index – ‘size index’ – is based on the low mAG-geminin and high FSC-W signals, which characterize cytokinetic cells. The size index scores for two distinct size ranges, named here ‘F1’ and ‘F2’ (top panel, dotted and straight gates). F1 is an index for cytokinetic cells with extreme width at the vertical dimension (during flow); these cells are for the most part 8-shaped. The F2 index captures cytokinetic cells with shape and dimensions closer to that of G2/M cells. Images depict DNA-stained L1210 cells sorted by F1 (top) and F2 (bottom) indices. (d) DNA contents of F1, F2 and asynchronous populations are shown. (a-c) Overall, cytokinetic cells can be distinguished from G1 cells by DNA content and cell size, and from G2/M cells by mAG-geminin level (G1 and G2/M populations are indicated by arrowheads). (a-c) Bivariate plots depicting indices of cytokinesis for L1210 cells treated 1 or 2 h with DMSO (control; top panels) or with 2 μ M pan-aurora kinase inhibitor VX-680 (bottom panels). Cells were stained with Hoechst 33342 prior to analysis. (e) Mean and standard deviation (SD) values for the four indices (a-c) are shown; $N=3$. Mean values are normalized to

control (DMSO). *P value < 0.05; **P value < 0.01. **(f)** An image of VX-680-treated cells (2 h). Cells were sorted based on F2-size index into media containing Hoechst 33342. **(g-h)** Bivariate plots depicting indices of cytokinesis for cells treated 2 h with DMSO or with 25 nM calyculin A (CaA). Cells were stained with Hoechst 33342 prior to analysis (g). Mean and standard deviation (SD) values for the four indices are shown (h); $N = 3$; *P value < 0.02. See Supplementary material for more information.

Figure 2. Inhibition of PP1/PP2A in late cytokinesis induces cytokinesis regression and tetraploidization. (a-b) Size index F1 allows label-free purification of late-cytokinesis cells by flow cytometry. The sorted cells exhibit fully ingressed furrow or intercellular bridge (a), reformed nuclei and decondensed chromatin (b). DIC (a) and confocal (b) images of L1210 cells are shown. (b) The cell is stained with Hoechst 33342 and transiently expresses lamin B1-mApple. (c-e) Late-cytokinesis L1210 cells were sorted into media containing either DMSO or CaA. (c) Image time series (DIC) demonstrating cytokinesis regression (CR) induced by 5 nM CaA. The black arrowhead points to a large membrane bleb typical of this process. Inlet images highlight intercellular bridges at t_0 . (d) Percentages of late-cytokinesis cells that completed cytokinesis regression (% of CR), were quantified over 3 h incubation with various doses of CaA. * P value < 0.001. (e) Time distributions of cytokinesis regression for each CaA dose are shown in matching colors. Mean (X), median (–) and four quantiles (Box and Whiskers) are depicted. Sample sizes (N) are indicated in matching colors (d-e). * P value < 0.02. (f-h) Late-cytokinesis HeLa cells were isolated based on size index. (f) Image times series (DIC) demonstrating cytokinesis regression in late-cytokinesis HeLa cells in response to 5 nM CaA. Inlet images highlight intense blebbing typically associated with cytokinesis regression in these cells. White arrow depicts a thin intercellular bridge. (g) Rate of cytokinesis regression in HeLa cells. Sample sizes (N) are indicated. * P value < 0.001. (h) Percentage of cytokinesis regression following 3 h treatment with DMSO, 1 nM CaA or 5 nM CaA. Sample sizes (N) are indicated. * P value < 0.01. (i) Late-cytokinesis L1210 cells expressing a GFP-tagged membrane marker (mem-GFP) were isolated using the doublet index. Cells were stained with Hoechst 33342 and SiR-tubulin (20 nM) pre- and post-sorting, respectively. Shown are merged- and single channel confocal images capturing two time points along cytokinesis regression induced by 5 nM CaA. Inlet magnified image highlights a typical early membrane expansion event at what used to be the cell's midbody. (j) Confocal image time series of a mem-GFP-expressing L1210 cell undergoing CaA-induced cytokinesis regression. DNA labeling (Hoechst 33342) reveals chromatin coalescence with no indications of chromatin recondensation. (k) An equivalent experiment with L1210 cells transiently expressing lamin B1-mApple demonstrates reorganization of the two sister nuclei.

Figure 3. Cytokinesis termination is secured by PP1/PP2A suppression of aurora kinases and cytokinesis regression. (a) Late-cytokinesis L1210 cells were sorted and treated with CaA or with the following kinase inhibitors: RO-3306 (inhibitor of CDK1), VX-680 (aurora A/B/C), AZD-1152 (aurora B), or BI2356 (PLK1). Percentage of complete cytokinesis regression (CR) at $t=3$ h are shown. (b-e) Plots depict percentage of cytokinesis regression in late-cytokinesis L1210 cells treated with 5 nM CaA and either RO-3306 (b) VX-680 (c), AZD-1152 (d) or BI2356 (e). (a-f) Percentage of cytokinesis regression induced by 5 nM CaA are shown for a comparison (adapted from Figure 1). Sample sizes (N) and drug concentration are indicated in matching colors. * P value < 0.05, ** P value < 0.01. (f) Image time series (DIC) demonstrating repression of CaA-induced cytokinesis regression in L1210 cells by VX-680 but not by BI2356. (g) A schematic of the regulation of cytokinesis termination. Aurora kinases and PP1/PP2A counteractivity and feedback mechanisms control mitotic progression and cell division. Here we show that by the time cells are about to conclude cytokinesis they still have inherent capacity to reverse cytokinesis. This phenomenon is executed by aurora kinases, and prevented by PP1/PP2A phosphatases.

Figure 4. Inhibition of aurora kinases induces cytokinesis failure, but not ‘cytokinesis regression’. (a) Schematic of possible fates of early mitotic cells under perturbation. (b-c) L1210 cells at G2/M were sorted and incubated with DMSO or 4 μ M of the aurora kinases inhibitor VX-680 for 6 h. (b) Image time series (DIC) of G2/M cells cultured with VX-680 or DMSO. Cells treated with VX-680 exhibit delayed entry to cytokinesis ($t=225$ min), cytokinesis failures and opening of the cleavage furrow (see white and black arrows). (c) Plots depicting the percentage of G2/M cells which either divided (Div), did not divide (No Div), or underwent cytokinesis failure (Cyt failure). Sample sizes (N) are indicated. (d) A representative image time series (DIC) showing no cytokinesis regression in late-cytokinesis L1210 cells incubated with 4 μ M VX-680. (e) A schematic demonstrating the different effects of CaA vs. VX-680 treatments and the importance of perturbation timing. Inhibition of aurora kinases before anaphase, blocks or delays entry into cytokinesis. Cells that do commence cytokinesis fail to conclude furrow ingression and eventually re-acquire the shape of a sphere (b). At late cytokinesis, inhibition of PP1/PP2A, but not of aurora kinases induces cytokinesis regression (d and Figure 3).

Materials and Methods

Cell culture

L1210 murine lymphoblasts, MOLT4 human T lymphoblasts, HeLa human epithelial cells were originally purchased from the ATCC; Cat#: CCL-219, CRL-1582, and CCL-2, respectively. A culture of MOLM14 were kindly provided by Assaf Bester (Israel Institute of Technology, Israel). L1210 cells stably expressing the mA-geminin cell cycle marker³⁰ or the mem-GFP membrane marker³¹ were previously described. HeLa cells stably expressing mA-geminin were generated by viral infection. To this end, mAG-geminin was cloned into Lentiviral vector carrying the Blasticidin resistance gene³⁰. Viral particles were generated in 293T cells. HeLa cells were incubated with media containing secreted viral particles (without further viral purification) for 24 h. Polybrene (8 µg/ml) was added to facilitate infection. Cells were washed and cultured in selection media containing 5 µg/ml Blasticidin. Stable cell lines used in this study originated from a single cell. L1210 and MOLT4 cell lines were maintained in Leibovitz's L-15 medium (Gibco; 21083-027). HeLa and MOLM13/14 cell lines were maintained in DMEM and RPMI medium, respectively (Biological Industries; #01-055-1A, #01-100-1A). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries; #040071), 2 mM Glutamine (Biological Industries; #03-020-1B), 1% of 100X penicillin-streptomycin solution (Biological Industries; #03-031-1B). L1210 and L1210-based cell lines were also supplemented with 0.2% of D-(+)-Glucose solution (Sigma Aldrich; #G8769). Cells were maintained at 37°C in a humidified atmosphere. HeLa, MOLM13 and MOLM14 were maintained in 5% CO₂ environment.

Small molecule inhibitors

The following drugs were used: Phosphatase inhibitors: Calyculin A (Abcam; #ab141784, Cell Signaling Technology; #9902S), okadaic acid (Abcam; #78111-17-8), and GSK2830371 (APEX BIO; #B1169); Kinase inhibitors: RO-3306 (Santa Cruz; #SC358700), VX-680 (Selleck Chemical; #S1048), AZD-1152 (Sigma-Aldrich; #SML0268), BI-2356 (Selleck Chemical; #S1109), MG132 (Calbiochem; #474790-5MG), and bortezomib (Sigma-Aldrich; #5043140001).

DNA delivery and staining

mAG-geminin expressing L1210 and HeLa cells were transfected with a plasmid coding for mApple-laminB1-10 (Addgene #54917) or Histone H2B-CFP (a gift from Yaon Shav-Tal, Bar Ilan University, Israel) using AMAXA Nucleofector 2b (Lonza) using Ingenio® Kit solution (Mirus Bio). Transiently transfected cells were processed for sorting after ~30-40 hrs. For DNA quantification, cells at 10^6 /ml concentration (+/- 15%) were incubated with 5 µg/ml Hoechst 33342 solution (Sigma-Aldrich; #B2261) for 30 min at 37° C. For imaging, a less stringent protocol was used.

Optical flow cytometry

Cell sorting and analysis by flow cytometry were all performed on BD FACSAria III. For DNA quantification, we used a 405 nm laser for excitation and a 450/50 nm bandpass filter for detection. For quantifying mAG-geminin level, we used a 488 nm laser for excitation and a 530/30 nm bandpass filter for emission. The 488 nm laser was also used for all light scatter measurements (default settings). FlowJo v10 software (FlowJo) was used for data processing and visualization. Phase-, Doublet-, and Size index for cytokinetic cells, as

well as gating strategy for sorting G1, G2/M, and cytokinetic cells are described in great details in the supplementary material section. Sorting of late-cytokinesis cells performed with a 130 μ m nozzle and minimal possible flow rate and pressure to minimize mechanical damage and premature daughter cell separation.

Live cell imaging

Sample processing for imaging. For most part, cells were sorted into μ -Slide 8 well glass bottom chamber slide (ibidi; #80827) containing 250-300 μ l media. Alternatively, cells were sorted into wells of an optically clear 96-well dish. Wells were filled with 100-150 μ l media. Microtubule of live cells were labeled with 20 nM SiR-tubulin (SPIROCHROME) and 10 μ M Verapamil (20 min, 37° C).

Time-lapse microscopy. Several microscope setups were used for live cell imaging. 1) Nikon eclipse TS100 inverted microscope equipped with 20X (NA: 0.4) and 40X (NA: 0.55) LWD objectives for phase imaging, a Nikon Digital-Slight DS-Fi1 camera, and a Nikon C-HGFI Intensilight light source. 2) Nikon Eclipse Ti-E inverted microscope equipped with 20X (NA: 0.45) and 40X (NA: 0.6) S plan Fluor ELWD objectives for differential interference contrast (DIC) and fluorescence imaging, Lumencor illuminator LED light source, a Zyla sCMOS camera (Andor Technology), and an Okolab incubator. Filter sets for DNA imaging: excitation 395/25 nm, emission 460/50 nm. Filter sets for mAG-geminin detection: excitation 470/24 nm, emission 530/30. 3) Zeiss Observer Z1 inverted microscope equipped with 20X Plan-Apochromate objectives (NA: 0.8), Lumencor SOLA light engine (light source), a Hamamatsu Orca CCD camera, and PeCon incubator. Filter sets for mAG-geminin detection: excitation 470/20, emission 514/5. 4) Leica DMI8 inverted microscope equipped with 20X (NA: 0.4) HC PL FLUOTAR L objectives for DIC and fluorescence imaging, Leica EL6000 light source, a Leica

DFC9000GT camera, and PeCon Incubator i8. Filter sets for mAG-geminin detection: excitation 490/12 nm, emission 525/36. 5) Etaluma lumascope 720 imaging system equipped with Olympus 10X (NA: 0.3) and 20X (NA: 0.5) OPLFLN objectives. 6) IncuCyte imaging system equipped with 10X IncuCyte Objective (NA: 0.3). Etaluma and IncuCyte imaging system was placed in external incubator for temperature and CO₂ control.

Confocal imaging. For confocal imaging, we used an inverted Leica DMI8 scanning confocal microscope, equipped with 63X (NA: 1.40) Plan apochromat oil objective, HyD detector, and Life Imaging Service (LIS) Incubator. Signal detection: DNA – excitation 405 nm laser, emission 418-462 nm; mem-GFP – excitation 488 nm laser, emission 495-556 nm; mApple-lamin B1 – excitation 552 nm laser, emission 563-615 nm; Sir-tubulin – excitation 638 nm laser, emission 645-723 nm. During imaging cells were maintained at 37° C and atmospheric (L1210 and MOLT4 cells) or 5% CO₂ (HeLa and MOLM14 cells) environment.

Image acquisition and processing. The following software were used: 1) NIS-Elements (Nikon TiE); 2) ZEN (Zeiss); 3) Lumaview (Etaluma); 4) LAS X (Leica Microsystems); and 5) ImageJ (Fiji). Confocal Z-stacks were generated from images taken at 0.2-0.585 µm intervals, and submitted to deconvolution with Hyvolution software. Deconvolved images were exported back into LASX and were then max projected into single x-y plane images

Quantification of cytokinesis regression in late-cytokinesis cells

Percentage of cytokinesis regression was determined based on time-lapse microscopy and image series of 5 to 10 min time intervals. Sample size represents the number of late-cytokinesis cells at t_0 that were successfully tracked for 3 h or until cytokinesis reversal was scored. Overall, a cytokinesis regression event was scored whenever late-cytokinesis daughter cells fully coalesced, *i.e.*, became spherical or near spherical. Percentage of

cytokinesis regression represents the cumulative fraction of fully coalesced cytokinetic cells from t_0 to t_N , and plotted in 20 min time intervals. Sample sizes for all experiments involving calyculin A, okadaic acid, and drug combinations integrates data from 2 to 5 measurements. Overall sample sizes in this study were exceptionally large by any standard as means of validation of truly unexpected phenomena.

Statistical and computational analysis

All plots were generated by Microsoft Excel. T-Test, χ^2 -Test, and F-Test were used for statistical analysis. We used Microsoft Excel software for T-Test and MATLAB® software (MathWorks) for χ^2 - and F-tests. Mathematical regression and F-Tests are detailed in the Supplementary information section.

Supplementary Material

for:

PP1/PP2A phosphatase activity ensures directional cell division by preventing cytokinesis regression

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699 **High-throughput flow cytometry analysis of cytokinesis failure in unattached cells;**
700 **concept and pipeline.**

701 We developed a pipeline for evaluation of cytokinesis in unattached cells. The method is
702 based on applying short-term biochemical perturbations in lymphoblasts using small-
703 molecule drugs, and scoring of cytokinetic cells by optical flow cytometry.

704 L1210 lymphoblasts are small, spherical (axial ratio of 1.01) and have a remarkable tendency
705 to grow only as singlets^{1,2}. These qualities are ideal for flow cytometry-based analyses and
706 cell size measurements^{3,4}. Furthermore, the average life cycle of L1210 cells is
707 approximately 11 h^{2,5}. Consequently, the fraction of dividing cells in steady-state culture
708 conditions is double that of mammalian cells with typical life cycles of 20-24 hrs.

709 We established and optimized three complementary flow cytometry-based indices for high-
710 throughput analysis of cytokinesis in lymphoblasts. The indices include the '**phase index**',
711 the '**doublet index**' and the '**size index**', described below. Each index is based on a
712 combination of two light scatter and/or fluorescence signals available in standard flow
713 cytometers. The method is demonstrated using L1210 murine lymphoblasts cells, but is
714 compatible with other cell types.

715 Monomeric Azami Green (mAG)-geminin is a fluorescent cell cycle marker regulated by the
716 E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C)⁶. The degradation of
717 mAG-geminin commences at the metaphase-to-anaphase transition and ends at the G1/S
718 transition of the following cell cycle. Thus, the expression levels of mAG-geminin during late
719 cytokinesis and in G1 are minimal. mAG-geminin-expressing L1210 cells were incubated
720 with DMSO (control) or 2 μ M VX-680 (inhibitor of aurora kinases) for 1 or 2 h, stained with
721 Hoechst 33342 (a cell-permeable chromatin dye) and analyzed by BD FACSAria III.

Phase index. The mAG-geminin/DNA bivariate plot classifies cytokinetic cells based on cell-cycle phase information (gated by a red square). Cells with 4N DNA that express basal mAG-geminin levels have passed anaphase but are yet to divide. Because cytokinesis commences simultaneously with anaphase, these cells are by definition in cytokinesis¹. The phase index does not rely on chemical labeling.

Doublet index. Flow cytometry provides fast and precise single-cell data. Exclusion of cell doublets and aggregates is therefore necessary for assay reliability. Pulse width, height and area of the DNA signal are informative optical metrics for scoring cell doublets⁷. The DNA-W/DNA-A bivariate plot is typically used for doublet discrimination (though equivalent gating strategies exist)⁷. Because of their remarkable sphericity, L1210 cells that have a fully ingressed cleavage furrow and the 8-shaped morphology typical of late cytokinesis acquire a near perfect 8-shaped morphology with an axial ratio of ~2. Cells exhibiting this geometry can be classified incorrectly as ‘doublets’ by flow cytometry. Surely, droplets categorized as doublets can carry cytokinesis cells¹. However, to what extent doublet discrimination introduces bias in analyses of cycling cells is unclear. As L1210 cells do not aggregate, we speculated that nearly any L1210 cell identified as a doublet is actually a single cell at late cytokinesis. VX-680 is a well-studied pan-aurora kinase inhibitor VX-680, which induces cytokinesis failure. Using this reagent, we demonstrated that the canonical index of ‘cell doublets’ is effectively an index of late-cytokinesis cells. The ‘Doublet index’ relies solely on DNA staining and is considerably more specific and sensitive than the Phase index because of its high selectivity for 8-shaped late cytokinesis cells (a full description of the doublet index is detailed in a complementary study co-submitted to *eLife*; Panet et al., 2021).

Size index. Approximation of cell size by light scatter parameters is accurate for spherical objects with homogenous texture. L1210 cells fall well within this category. Cytokinetic cells are, by definition, the largest in an asynchronous cell population. However, unlike the similarly-sized G2/M cells, their mAG-geminin level is low, meaning these two subpopulations are optically distinguishable. Importantly, the size index scores two distinct size ranges, named here 'F1' and 'F2', both of which represent cells with 4N DNA. F1 is an index of cytokinetic cells with extreme width at the vertical dimension (during flow). These cells are for the most part 8-shaped. The F2 index captures cytokinetic cells with shape and dimensions closer to that of G2/M cells. Being free from chemical labelling and highly informative, we extensively used the size index for both sorting and analysis of cytokinetic cells.

At this juncture, it is important to note that each of the abovementioned indices are time invariant in steady-state culture conditions owing to the constant flux of cells that enter to and exit from cytokinesis. Chemical perturbations may shift one or both fluxes in ways that effectively change index values. For example, failure in furrow ingression is likely to reduce the doublet index whereas halted abscission will increase the fraction of 8-shaped cells.

Summary. Cytokinesis is under-studied in unattached cells. We established three different flow cytometry indices for identifying cytokinetic cells, each with advantages and limitations. Together, however, these indices provide complementary information on cytokinesis and cell division. The methodology is simple, handy, optimal for both low- and high-throughput assays, and can be applied also to adherent cells. Unlike the classic G2/M index, cytokinesis indices highlight a brief period within the cell — from anaphase to abscission. The fraction of cells in this phase is low, so short-term perturbations can

generate considerable shifts in index values that are noticeable directly from the bivariate plots, even without computation. Owing to the pleiotropic effects of many drugs (whether they are specific or not), this feature of the method simplifies cytokinesis analysis, and improves precision and data interpretation.

Supplementary Figures and Figure legends

Supplementary Figure 1

Figure S1. Pan-aurora kinase inhibitor VX-680 induces cytokinesis failure in L1210 mouse lymphoblasts. mAG-geminin-expressing L1210 cells were incubated with DMSO or 2 μ M VX-680. **(a)** DNA content analysis (Hoechst 33342) of cells treated 2 h with DMSO or VX-680. **(b)** A bivariate plot showing a gating strategy for purifying cells at G2-phase and mitosis (G2/M) phases cells without chemical labeling. **(c)** G2/M cells were sorted into fresh media containing DMSO or 2 μ M VX-680 and imaged for 5 h. A representative image series is shown. Cells are tracked by arrows with matching colors. A cell undergoing furrow regression induced by VX-680-mediated cytokinesis failure is framed.

Figure S2. Cytokinesis indices reveals impaired cytokinesis following short-term inhibition of PP1/PP2A. (a) mAG-geminin-expressing L1210 cells were incubated 1 h with DMSO or 25 nM of the PP1/PP2A inhibitor Calyculin A (CaA) and analyzed by flow cytometry. Depicted, bivariate plots showing ‘Phase index’, ‘Doublet index’ and ‘Size indexes F1 and F2’ for cytokinetic cells (see Figure 1 for more information). **(b)** Mean and SD values of each index are plotted. Mean values are normalized to control (DMSO). $N = 3$; * P value < 0.01 . ns (non-significant): P value > 0.05 .

Supplementary Figure 3

Figure S3. Calyculin A blocks division of L1210 cells. L1210 cells at the G2/M phase were sorted (Figure S1b) into media containing either 5 nM CaA or DMSO. Cell division was monitored by microscopy (x20 lens). The percentage of cells that divided within 3 h are shown. * P value < 0.01.

Supplementary Figure 4

Figure S4. Confocal images of late-cytokinesis L1210 cells. Late-cytokinesis L1210 cells expressing a GFP-tagged membrane marker (mem-GFP) were isolated using the doublet index (see Figure 1b). Cells were stained with Hoechst 33342 and SiR-tubulin (20 nM) pre- and post-sorting, respectively. Shown are merged- and single channel confocal images (x63) processed by maximum intensity projection.

Supplementary Figure 5

L1210

Figure S5. Reproducibility of CaA-induced cytokinesis regression. Late-cytokinesis L1210 cells were isolated by 'Size index'-based sorting (see Figure 1 for more information). Cells were incubated in media supplemented with 5 nM CaA, and imaged for 180 min. Percentages of late-cytokinesis cells undergoing cytokinesis regression (% CR) are shown in 20 min time intervals. Plots depict data from three independent experiment spanning over 14 months through which three different batches of CaA were used. Mean values are also shown (black line). Batch number, date of experiment, and sample size are shown in matching colors.

Supplementary Figure S6

Figure S6. PP1/PP2A inhibition by CaA does not induce cell fusion. (a) A bivariate plot highlighting L1210 cells with minimal mAG-geminin and FSC-W signals (black square gate). These cells are at the G1 phase of the cell cycle. **(b)** L1210 cells at G1 were sorted and incubated with 25 nM CaA. Cells were monitored for 4 hrs. Image time series of two representative fields of view are shown (DIC; X20 lens). Cells in close proximity are tracked by arrowheads with matching colors. Over 100 cells were monitored, none of which coalesced.

856 **Supplementary Figure S7**

Asynchronous L1210 cells

857

858 **Figure S7. *CaA-induced cytokinesis regression is independent of cell sorting.*** Asynchronous L1210 cells were
859 cultured in media containing 5 nM CaA. Cells were monitored for 3 hrs. Random cells exhibiting fully ingressed
860 furrow or intercellular bridge at t_0 were tracked. Image time series of a representative field of view is shown
861 (DIC; X20 lens). Cells undergoing cytokinesis regression are tracked by arrowheads with matching colors (top).
862 A section of the field of view (framed) was magnified for better visualization. Two cells undergoing cytokinesis
863 regression are indicated by white arrows.

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865

Figure S8. Cytokinesis regression in late-cytokinesis HeLa human epithelial cells. (a) Cytokinesis indices reveals impaired cytokinesis in HeLa cells following short-term inhibition of PP1/PP2A. mAG-geminin-expressing HeLa cells were incubated 2 h with DMSO or 5 nM of the PP1/PP2A inhibitor CaA and analyzed by flow cytometry. Depicted, bivariate plots showing ‘Phase index’, ‘Doublet index’ and ‘Size indexes F1 and F2’ for cytokinetic cells (see Figure 1 for more information). **(b)** Isolating late-cytokinesis HeLa cells. mAG-geminin expressing HeLa cells were analyzed by flow cytometry. An mAG-geminin/FSC-W bivariate plot showing the gate for ‘Size index’ (black square), which we use for sorting late-cytokinesis cells. A brightfield image of post-sort HeLa cells (X10 lens) demonstrates the selective enrichment of late-cytokinesis HeLa cells by this method. **(c)** Reproducibility of CaA-induced cytokinesis regression in HeLa cells. Late-cytokinesis HeLa cells were isolated by ‘Size index’-based sorting (see b). Cells were incubated in media supplemented with 5 nM CaA, and imaged for 200 min. Percentages of late-cytokinesis cells undergoing cytokinesis regression (% CR) are shown in 20 min time intervals. Plots depict data from three independent experiment spanning over 14 months through which three different batches of CaA were used. Mean values are also shown (black line). Batch number, date of experiment, and sample size are shown in matching colors. **(d)** mAG-geminin-expressing HeLa cells cultured in media containing 5 nM CaA were monitored for 1-3 hrs. Random cells exhibiting fully ingressed furrow or intercellular bridge at t_0 were tracked. Image time series of cells undergoing cytokinesis regression are shown (X20 lens). DIC and mAG-geminin fluorescent channels were merged for presentation. Basal levels of mAG-geminin mirror the advanced state from which cytokinesis can be reversed. A cell undergoing cytokinesis regression is tracked by white arrows.

887 **Supplementary Figure S9**

888

889 **Figure S9. Cytokinesis regression in late-cytokinesis human lymphoblasts.** MOLM13, MOLM14 and MOLT4
 890 human lymphoblasts cultured in media containing 5 nM CaA were monitored for 1-3 hrs. Random cells
 891 exhibiting fully ingressed furrow or intercellular bridge at t_0 were tracked. Image time series of cells
 892 undergoing cytokinesis regression are shown (white arrows; DIC; X20 lens).

Supplementary Figure S10

Figure S10. Inhibition of PP1/PP2A by okadaic acid induces cytokinesis regression in late-cytokinesis L1210 cells. Late-cytokinesis L1210 cells were isolated by 'Size index'-based sorting (see Figure 1 for more information). Cells were incubated in media supplemented with DMSO, 1- or 5 μ M okadaic acid (OA), and imaged for 180 min. **(a)** Percentages of cytokinesis regression (%CR) were quantified in 20 min time intervals. * P value < 0.001 . **(b)** Time-distribution of cytokinesis regression is depicted by a box and whiskers plot. Mean (X), median (–) and four quantiles are shown. * $P < 0.01$. **(c)** An equivalent control experiment performed in the presence of GSK2830371 Wip1 phosphatase inhibitor. Percentage of cytokinesis regression induced by 5 μ M GSK2830371 are shown for $t = 3$ h in comparison to DMSO and OA. * P value < 0.01 . Sample sizes (N) indicated in (c) applied to all three plots (see matching colors). **(d)** An image-time series demonstrates profound and accelerated cytokinesis regression in response to 5 μ M OA (DIC; X10 lens).

Supplementary Figure S11

Figure S11. Okadaic acid induces cytokinesis regression in late-cytokinesis HeLa cells. Late-cytokinesis HeLa cells were isolated by 'Size index'-based sorting (see Figure 1 for more information). Cells were incubated in media supplemented with DMSO or 2.5 μ M okadaic acid (OA), and imaged for 3 hrs. **(a)** Percentages of cytokinesis regression (%CR) were quantified in 20 min time intervals. **P* value << 0.001. **(b)** Time-distribution of OA-induced cytokinesis regression. Mean (X), median (–) and four quantiles (box and whiskers) are shown. **P* < 0.01. **(c)** An equivalent control experiment performed in cells treated with 5 μ M GSK2830371 Wip1 phosphatase inhibitor. Percentage of cytokinesis regression induced by GSK2830371 are shown for *t* = 3 h in comparison to DMSO and 2.5 μ M OA. **P* value < 0.01. Sample sizes (*N*) indicated in (c) applied to all three plots (see matching colors). **(d)** An image-time series of HeLa cells undergoing cytokinesis regression in response to 2.5 μ M OA (Brightfield; X20 lens).

919 **Supplementary Figure S12**

920 % of FR at $t_{3h} = 40.17\%$ ($N=1023$)

921 **Figure S12. Cytokinesis regression in cells stained with Hoechst 33342.** Asynchronous L1210 cells were
 922 stained with Hoechst 33342, incubated in media supplemented with 5 nM CaA, and imaged for 180 min. An
 923 image time series of a random late-cytokinesis cell undergoing cytokinesis regression is shown. DIC- and
 924 fluorescent (405 nm excitation) channels are shown separately and together (merged; X40 lens).

Supplementary Figure S13

Figure S13. Indications for nuclear coalescence following CaA-induced cytokinesis regression. mAG-geminin-expressing L1210 **(a)** and HeLa **(b)** cells were electroporated with mApple-lamin B1 and cultured for ~30 hrs. Late-cytokinesis HeLa cells expressing mApple-lamin B1 were isolated by ‘Size index’-based sorting. Cells were incubated with 10 nM CaA, and imaged by a confocal microscope (X63 lens). Maximum projection images and an entire set of deconvoluted z sections at t=43 min are shown for an L1210 cell undergoing cytokinesis regression (a). Separate and merged fluorescent and brightfield images of a binucleated HeLa cell following CaA-induced cytokinesis regression are shown in b.

Supplementary Figure S14

Figure S14. PP1/PP2A inhibition induces cytokinesis regression independent of chromatin bridges. (a) L1210 cells were fixed in 4% paraformaldehyde and labeled with DAPI. Late-cytokinesis cells were sorted (see Figure 1) and imaged. Single-channel and merged confocal images are shown. Inter-cellular bridges were magnified (farmed images). One out of 18 cells depicted chromatin bridges (green signal; labeled by white arrow). **(b)** L1210 cells were treated 10 min with 5 nM calyculin A (CaA), fixed and DNA labeled. Late-cytokinesis cells were sorted and processed as described in (a). A cell in advanced stage of cytokinesis regression is shown at the bottom. **(c)** Late-cytokinesis L1210 cells transiently expressing Histone H2B-CFP were sorted into fresh media, treated with 5 nM CaA and imaged.

948 **Supplementary Figure S15**

949

950 **Figure S15. Cytokinesis regression in late cytokinesis is not induced by mitotic kinase inhibitors.** Late-
 951 cytokinesis L1210 cells were isolated by 'Size index'-based sorting. Cells were incubated in media
 952 supplemented with the mitotic kinase inhibitors: RO-3306 (inhibitor of CDK1), VX-680 (aurora A/B/C), AZD-
 953 1152 (aurora B), and BI-2356 (PLK1). Cells were monitored for 3 h by DIC microscopy (X20 lens).
 954 Representative image time series are shown. Arrowheads with matching colors were added to simplify
 955 tracking of moving cells.

Supplementary Figure S16

Figure S16. Cytokinesis regression induced by PP1/PP2A inhibition is repressed by inhibition of aurora kinases. Late-cytokinesis L1210 cells were isolated by ‘Size index’-based sorting, and incubated with 5 nM CaA and each of the depicted mitotic kinase inhibitors: RO-3306 (inhibitor of CDK1), VX-680 (aurora A/B/C), AZD-1152 (aurora B), and BI-2356 (PLK1). Cells were monitored for 3 h by DIC microscopy (X20 lens). Representative image time series are shown. Arrows depict late-cytokinesis cells undergoing cytokinesis regression.

Supplementary Figure S17

Figure S17. Inhibition of aurora kinase induces cytokinesis failure but not cytokinesis regression in late cytokinesis. (a) L1210 cells at G2/M were sorted (see gating strategy in Figure S1b) and incubated for 3 h with DMSO or 4 μM VX-680. Cells were recorded by DIC microscopy (X20 lens). Image time series of representative fields of view are shown for each treatment. Arrow heads depict cells undergoing cytokinesis failure. (b) Late-cytokinesis L1210 cells were isolated by 'Size index'-based sorting. Cells were incubated in media supplemented with DMSO or 4 μM VX-680 and monitored for 3 h by DIC microscopy (X20 lens). Representative image time series are shown. Late-cytokinesis cells are tracked by arrows with matching colors.

F test for regression

Statistical significance associated with level and kinetics of cytokinesis regression was determined using regression following the logistic function equation:

$$P = \frac{Asym}{1 + e^{\frac{x_{mid}-t}{scal}}}$$

where 'Asym' is the carrying capacity, 'xmid' is the x value at the inflection point of the logistic curve, and 'scal' is a scaling parameter for the x-axis. The function is characterized by a sigmoidal (s-shaped) relationship. Depicted below, are examples for three Specific models (left plot), each generated by a three-parameter-regression, and a General model (right plot) of percentage of cytokinesis regression calculated for L1210 cells treated with 5-, 10- and 50 nM CaA (Figure 2).

We calculate F value according to the following formula:

$$F = \frac{\left(\frac{RSS_1 - RSS_2}{p_2 - p_1} \right)}{\left(\frac{RSS_2}{n - p_2} \right)}$$

where RSS_i is the residual sum of squares of model i , p_i is the number of parameters of model i , and n is the sample size. For example, in the data presented above RSS_1 equal 7.8542, RSS_2 equal 0.2272, p_2 equal 9, p_1 equal 3, $n=456$, F equal 2500. We calculate p -value according to the following formula: $p = 1 - \text{fcdf}(F, p_2 - p_1, n - p_2)$. Fcdf stands for 'F cumulative distribution function'. The p value of the experiment depicted above is <0.001 . If there is a statistical significance between the general model and the specific

model, we calculate new F and p -value between each pair of models with matching parameters. See the plot below for an example.

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Supplementary movie legends

Supplementary Movie 1: Supplemental to Figure 2c, top panels. A late-cytokinesis L1210 cell (#1) undergoing cytokinesis regression induced by calyculin A

Supplementary Movie 2: Supplemental to Figure 2c, mid panels. A late-cytokinesis L1210 cell (#2) undergoing cytokinesis regression induced by calyculin A

Supplementary Movie 3: Supplemental to Figure 2f. Two late-cytokinesis HeLa cells undergoing cytokinesis regression induced by calyculin A

Supplementary Movie 4: Supplemental to Figure S10. Late-cytokinesis L1210 cells undergoing cytokinesis regression induced by okadaic acid

Supplementary Movie 5: Supplemental to Figure 3b. Late-cytokinesis L1210 cells undergoing cytokinesis regression induced by calyculin A

Supplementary Movie 6: Supplemental to Figure 3b. Cytokinesis regression in late-cytokinesis L1210 cells incubated with calyculin A and the CDK1 inhibitor RO-3306

Supplementary Movie 7: Supplemental to Figure 3c. Inhibition of aurora kinase by VX-680 represses calyculin A-induced cytokinesis regression in L1210 cells

1046 **Supplementary Movie 8:** Supplemental to Figure 3d. Inhibition of aurora B by AZD-1152

1047 represses calyculin A-induced cytokinesis regression in L1210 cells

1048

1049 **Supplementary Movie 9:** Supplemental to Figure 3e. Inhibition of PLK1 by BI2356 facilitates

1050 calyculin A-induced cytokinesis regression in L1210 cells

1051

1052 **Supplementary Movie 10:** Supplemental to Figure 4b. Cytokinesis failure in G2/M L1210

1053 cells induced by VX-680

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